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APPLICATION NUMBER: *60/384,228*
FILING DATE: *May 30, 2002*
RELATED PCT APPLICATION NUMBER: *PCT/US03/16961*

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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. EL608110618US

INVENTOR(S)

Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Richard N.	Kolesnick	New York, New York
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☐ Additional inventors are being named on the ___ separately numbered sheets attached hereto

TITLE OF THE INVENTION (280 characters max)

Anti-Kinase Suppressor Of Ras, A Therapeutic Strategy In Ras Mediated Tumorigenesis

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

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ENCLOSED APPLICATION PARTS (check all that apply)

[X] Specification Number of Pages: 16
[X] Drawing(s) Number of Sheets: 10
☐ Application Data Sheet. See 37 CFR 1.76
☐ CD(s), Number
[X] Other (specify): 1 page summary

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

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Respectfully submitted,

SIGNATURE [signature] Date 5/30/02

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The Patent and Trademark Office stamping
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APPLICANT: Kolesnick, *et al.*
TITLE: Anti-Kinase Suppressor of ras, a therapeutic strategy in Ras mediated tumorigenesis

Comprising:
pages provisional application including 1 page of Abstract, 16 pages of description, and 10 sheets of drawings;

1 pages of Provisional Application Transmittal
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ANTI-KINASE SUPPRESSOR OF RAS, A THERAPEUTIC STRATEGY IN RAS MEDIATED TUMORIGENESIS

Inventors: Richard N. Kolesnik, Ph.D.
Hongmei R. Xing, Ph.D.

Location: Memorial Sloan Kettering Cancer Center
New York

Summary of the Invention

These studies demonstrate that mammalian KSR integrates signaling through the EGFR/Ras/MAPK signaling module. That EGFR, Ras and KSR are on the same signaling pathway in mammalian cells is supported by the unusual hair follicle phenotype manifested in EGFR knockout mice and recapitulated in the KSR knockout, by the attenuation of EGF-induced MAPK signaling in MEFs, and by the abrogation of EGFR-/Ras-mediated tumorigenesis in multiple experimental models. Further, genetic and pharmacologic approaches identified KSR as required for various aspects of tumorigenesis *in vitro* and *in vivo*. *In vitro*, loss of KSR function reduced proliferation of MEFs, A431 and MCF-7 cells, abrogated Ras-mediated MEF transformation, and attenuated A431 and MCF-7 cell invasion. *In vivo*, inactivation of KSR antagonized *v-Ha-Ras*-mediated tumor formation and growth of an established EGFR-driven tumor that requires wild type Ras for neoplastic progression. As in *C. elegans*[2,3], KSR appears dispensable, for the most part, for normal development, but required when increased signaling through the EGFR/Ras pathway is necessary, as occurs acutely in response to EGF stimulation or chronically in Ras-mediated tumors, suggesting the possibility that pharmacologic inactivation might yield a therapeutic gain. Supporting this proposal further, results obtained on human pancreatic cancer cells PNCA1, an other model of Ras-mediated tumorigenesis (Fig. 4-5) shows a significant inhibition of cell proliferation and cell invasion confirm the use of KSR-AS ODNs as a therapeutic approach in K-Ras mediated tumorigenesis.

Deficiency of kinase suppressor of Ras prevents oncogenic Ras signaling in mice

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Abstract

In *Drosophila melanogaster* and *Caenorhabditis elegans*, Kinase Suppressor of Ras (KSR) positively modulates Ras/mitogen-activated protein kinase (MAPK) signaling either upstream of or parallel to Raf[1-3]. The precise signaling mechanism of mammalian KSR, and its role in Ras-mediated transformation, however, remains uncertain. Utilizing

cells markedly overexpressing recombinant KSR, some groups reported KSR inhibits MAPK activation and Ras-induced transformation[4-6] while others observed enhancing effects[7-10]. Evidence suggests these discrepancies reflect gene dosage effects[11]. To gain insight into KSR function *in vivo*, we generated mice homozygous null for KSR. *ksr*$^{-/-}$ mice are viable and without major developmental defects. Newborn mice, however, display a unique hair follicle phenotype previously observed in EGFR-deficient mice. Embryonic fibroblasts from *ksr*$^{-/-}$ animals were defective in EGF activation of the MAPK pathway, and displayed diminished proliferative potential and impaired Ras-dependent transformability. Tumor formation in Tg.AC mice, resulting from skin-specific *v*-Ha-*ras* expression, was abrogated in the *ksr*$^{-/-}$ background. Moreover, pharmacologic KSR inactivation via KSR antisense oligonucleotides attenuated EGFR-driven, Ras-mediated proliferation and invasion of A431 epidermal carcinoma cells *in vitro*, and abrogated neoplastic progression of A431 xenografts in nude mice. Thus, genetic and pharmacologic evidence suggests KSR transduces some forms of EGFR-/Ras-mediated neoplasia, which may be potentially targeted by anti-KSR therapeutic strategies.

Results and Discussion

To investigate the *in vivo* function of KSR in mammals, we targeted the mouse *ksr* locus to obtain mice deficient in KSR expression. *ksr*$^{-/-}$ mice were generated by homologous recombination in embryonic stem (ES) cells using the pF9 targeting vector shown in Fig. 1*a*. The targeted region included the starting methionine (ATG codon at nt 83 in *ksr* cDNA) and the following 74 amino acids encompassing 85% of the KSR unique CA1 domain. Two targeted ES clones (Fig. 1*b*) were microinjected into C57BL/6 blastocysts and both resulted in chimeric mice that transmitted the mutated *ksr* allele through to the germline. Crosses of the *ksr*$^{+/-}$ mice generated progeny with genotypes of the expected Mendelian frequencies. A PCR-based screening strategy was developed to detect both the wild-type (wt) and mutated alleles from mouse genomic DNA (Fig. 1*c*).

As previously reported[12], Northern blot analysis revealed wt KSR transcripts of 6.4 and 7.4 kb. The smaller transcript was detected by embryonic day 7, while the larger transcript was observed from day 11 on (Fig. 1*d*). In the adult, numerous tissues expressed *ksr* transcripts including heart, spleen, lung, thymus, and brain (Fig. 1*e*). Kidney displayed little if any *ksr* mRNA, while the larger transcript was restricted to brain. The existence of this larger mRNA was recently reported by Morrison and co-workers to represent a splice variant of murine KSR1, named B-KSR1[12]. Importantly, *ksr*$^{-/-}$ mice did not express detectable levels of either *ksr* mRNA in any tissue tested (Fig. 1*e*). KSR1 and B-KSR1 proteins were also not detected by Western blot analysis in tissues or in mouse embryo fibroblasts (MEFs) from *ksr*$^{-/-}$ mice (Fig. 1*f*). The lack of KSR was also confirmed by RT-PCR analysis with primers specific for the 3'-UTR of *ksr* cDNA (not shown). Our data thus suggest that replacement of the 5' region of *ksr* including the start coding site and most of the CA1 domain successfully abolished expression of both murine KSR forms.

KSR knockout mice were viable and fertile, with no major developmental defects. No gross histologic abnormalities of the major organs were apparent in young mice or in adults up to one year of age. Animal weight, behavior and brood size were also unaffected in the KSR knockout. However, histologic examination of the skin of 10-day-old *ksr*$^{-/-}$ mice revealed noticeably fewer hair follicles, which were disorganized in dermal location (depth) and orientation (direction), and manifested asynchronous growth (Fig. 2*a* *vs.* 2*b,c*). Further, a significant proportion displayed a serpentine morphology (Fig. 2*b*). In other follicles, the inner root sheath separated from the hair shaft, resulting in

formation of blisters or cysts (Fig. 2*c*). Strikingly, this phenotype closely resembles that found in the skin of EGFR-deficient mice[13] (Fig. 2*d*). Grossly, *egfr*$^{-/-}$ mice display short, wavy pelage hair and curly whiskers during the first weeks of age, with pelage and vibrissa hairs becoming progressively sparser and atrophic over time, eventually leading to alopecia[13]. Although these gross phenotypes were not seen in *ksr*$^{-/-}$ mice, increased alopecia and sparse hair growth were observed following treatment with the phorbol ester 12-O-tetradecanoylphorbol 13-acetate (TPA) compared to similarly treated *ksr*$^{+/+}$ controls (not shown). The manifestation of this unique hair follicle phenotype by both knockouts supports the contention that EGFR and KSR might be on the same pathway in mice.

To further elucidate the effect of KSR disruption on activation of the EGFR/MAPK pathway, we generated MEFs from *ksr*$^{+/+}$ and *ksr*$^{-/-}$ littermates and evaluated their response to low, mitogenic doses of EGF (0.05-1.0 ng/ml). While there was no difference in EGFR autophosphorylation between *ksr*$^{+/+}$ and *ksr*$^{-/-}$ MEFs (not shown), *ksr*$^{-/-}$ MEFs displayed significant reduction in MEK1 and MAPK (ERK1/2) activation upon EGF stimulation, as detected using phospho-specific antibodies (Fig. 3*a*). These deficits could be partially overcome at higher EGF doses (not shown), consistent with activation of the MAPK pathway by alternative mechanisms[14]. Consistent with reduction in signaling through the MAPK pathway, which provides proliferative signals, we observed a 50% reduction in growth rate in *ksr*$^{-/-}$ MEFs (Fig. 3*b*).

To determine the potential impact of KSR inactivation in Ras-mediated transformation, c-Myc and Ha-*ras*V12 constructs were transduced into *ksr*$^{+/+}$ and *ksr*$^{-/-}$ early-passage MEFs using high-titer retroviruses, and the ability to grow as colonies in soft agar was assessed as described[15]. While *ksr*$^{+/+}$ fibroblasts did not form colonies in soft-agar, they did so in the presence of Myc and Ras oncogenes (not shown). In contrast, *ksr*$^{-/-}$ MEFs could not be transformed by Ha-*ras*V12, even though they were immortalized by c-Myc. Taken together, all these results show that inactivation of KSR by genetic deletion attenuates signaling through the EGFR/Ras/MAPK pathway.

Since *ksr*$^{-/-}$ mice showed a defect in normal development of the hair follicle, presumably via impairment of EGFR signaling, we examined the role of KSR in gain-of-function Ras in the skin. For these studies, we employed Tg.AC mice, which harbor oncogenic *v*-Ha-*ras* fused to the ζ-globin promoter[16-18], a standardized model for the study of two-stage skin carcinogenesis. The *v*-Ha-*ras* transgene of Tg.AC mice is transcriptionally silent until induced in latent neoplastic cells (putative stem cells) closely

associated with the outer root sheath cells of the hair follicle[19], a site consistent with our localization of KSR in mouse skin (not shown). Tg.AC mice (in FVB/N strain background) were crossed with *ksr*$^{-/-}$ mice (in a mixed C57BL/6:129sv background). F1 offspring heterozygous for the *ksr* gene were then interbred to obtain F2 offspring carrying the *v*-Ha-*ras* transgene in the *ksr*$^{+/+}$ and *ksr*$^{-/-}$ background. To determine if disruption of *ksr* might influence tumorigenesis in this model, we topically treated the dorsum of F2 mice twice weekly for 15 weeks with vehicle (acetone), or with 5 μg of TPA. Animals were monitored for development of skin malignancies for 20 weeks.

Initial control studies using RT-PCR to detect the *v*-Ha-*ras* transgene mRNA showed that loss of KSR function in *ksr*$^{-/-}$ mice had no impact on TPA-induced expression of the oncogenic *v*-Ha-*ras* transgene in the skin (Fig. 3*c*). However, 70% of Tg.AC transgenic mice in a *ksr*$^{+/+}$ background developed papillomas, while only 10% in a *ksr*$^{-/-}$ background displayed papillomas (Fig. 3*d*). The average number of papillomas in our study was 2-4 per mouse in each group. These studies with Tg.AC mice demonstrate that genetic inactivation of KSR prevents EGFR-/Ras-mediated skin tumorigenesis. Further, these studies suggest that KSR might serve as a target for pharmacologic intervention in Ras-mediated neoplasias.

To develop a potential therapeutic approach to KSR inactivation, we generated KSR-specific phosphorothioate antisense (AS) oligodeoxynucleotides (ODNs; see Methods). These studies employed the A431 epidermal carcinoma cell line in which tumor growth is driven through Ras by a 100-fold excess of activated EGFR/HER1 (10^7 receptor/cell)[20,21]. *In vitro*, KSR-AS ODN treatment attenuated A431 cell proliferation (Fig. 3*e*, $p<0.05$ *vs.* Control ODN at 200 nM) and invasion through matrigel (Fig. 3*e*, $p<0.05$ *vs.* Control ODN at 500 nM) in a dose-dependent fashion. In contrast, Control ODN (Fig. 3e), which lacks homology to any mammalian gene[22], or KSR-sense or mismatch KSR-AS ODNs (not shown), were ineffective. Similar results were obtained with MCF-7 mammary carcinoma cells (not shown). I*n vivo*, continuous infusion of KSR-AS ODN attenuated A431 tumor growth by 80% (Fig. 3*f*), without apparent toxicity (weight loss, behavioral alteration, organomegaly, inflammation, bleeding), consistent with the known lack of toxicity of this therapeutic approach[23]. In contrast, continuous infusion of vehicle alone (saline), Control ODN, or KSR-sense ODN exhibited no significant effect on A431 tumor growth (Fig. 3*f* and not shown). Similar results were obtained when treatment was started with established tumors of 150 mm^3 (not shown).

In summary, these studies demonstrate that mammalian KSR integrates signaling through the EGFR/Ras/MAPK signaling module. That EGFR, Ras and KSR are on the same signaling pathway in mammalian cells is supported by the unusual hair follicle phenotype manifested in EGFR knockout mice and recapitulated in the KSR knockout, by the attenuation of EGF-induced MAPK signaling in MEFs, and by the abrogation of EGFR-/Ras-mediated tumorigenesis in multiple experimental models. Further, genetic and pharmacologic approaches identified KSR as required for various aspects of tumorigenesis *in vitro* and *in vivo*. *In vitro*, loss of KSR function reduced proliferation of MEFs, A431 and MCF-7 cells, abrogated Ras-mediated MEF transformation, and attenuated A431 and MCF-7 cell invasion. *In vivo*, inactivation of KSR antagonized v-Ha-*Ras*-mediated tumor formation and growth of an established EGFR-driven tumor that requires wild type Ras for neoplastic progression. As in *C. elegans*[2,3], KSR appears dispensable, for the most part, for normal development, but required when increased signaling through the EGFR/Ras pathway is necessary, as occurs acutely in response to EGF stimulation or chronically in Ras-mediated tumors, suggesting the possibility that pharmacologic inactivation might yield a therapeutic gain. Consistent with this proposal, studies are currently underway to extend the use of KSR-AS ODNs to other models of Ras-mediated tumorigenesis (Xing and Kolesnick, unpublished).

Methods

Gene targeting. Mouse *ksr* genomic DNA clones were isolated by screening a λFixII phage library prepared from mouse strain 129/sv (Stratagene, La Jolla, MA) using the 5' coding region (nt 1-786) of mouse *ksr* cDNA (Genbank accession # U43585) as a probe. The targeting vector pF9 was constructed by inserting a 2.5-kb *Spe*I-*Sma*I fill-in fragment from the 5' end of the mouse *ksr* genomic clone into the *Not*I fill-in site of pPGK-NTK vector (a gift from Dr. Frank Sirotnak). A 6.3-kb *Spe*I-*Hind*III fill-in fragment from the 3' downstream region of the mouse *ksr* genomic clone was inserted into the vector at the *Cla*I fill-in site. The resulting plasmid was linearized with *Kpn*I and electroporated into 129/Sv-derived W9.5 ES cells (Chrysalis DNX Transgenic Sciences, Princeton, New Jersey). Two hundred G418/Gancyclovir-resistant ES cell clones were analyzed by Southern blot using a 0.6 kb *Bgl*II-*Spe*I probe derived from genomic sequences located immediately outside (5') those present in pF9. This probe hybridizes to a 5.7-kb DNA fragment for the wt *ksr* allele and a 3.1-kb fragment from the disrupted allele. Heterozygous ES cells were microinjected into blastocyst-stage C57BL/6 mouse embryos at the Sloan-Kettering Institute's Transgenic Core Facility. Injected blastocysts were then transplanted into the uterus of pseudopregnant C57BL/6 mice. Chimeric males were crossed to C57BL/6 females. Germline transmission was monitored by Southern blot in agouti F1 offspring. For mouse genotyping, genomic DNA was isolated from mouse tails with the DNeasy kit (Qiagen Inc., Valencia, CA) and was either digested with *Bgl*II and *Xho*I and examined by Southern blot as for ES cells, or analyzed by PCR amplification with two sets of primers. Primers for the wt allele were derived from the cDNA sequence of mouse *ksr* CA1 domain: upstream primer, 5'-TATCTCCATCGGCAGTCT-3', downstream primer, 5'- TCGACGCTCACACT TCAA-3'. The primers for the mutant allele were from the sequence of the neomycin phosphotransferase gene: upstream primer, 5'-CTGACCGCTTCCTCGTG-3'; downstream primer, 5'-ATAGAGCCCACCGCATCC-3'. The size of the expected product is 493-bp for the wt and 312-bp for the disrupted allele. Standard PCR conditions were employed: initial denaturation of 5 min at 94°C, followed by 30 cycles with annealing at 56°C, extension at 72°C, and denaturation at 94°C, all for 30 sec.

Northern and western blot analysis. Poly A^+ RNA was prepared from adult mouse tissues using the Oligotex kit from Qiagen Inc. (Valencia, CA). The blots were hybridized with a specific ^{32}P-labeled probe corresponding to the CA2-CA4 domains of murine *ksr* cDNA (1.47-kb). For embryonic tissues, we used a Mouse Embryo MTN Blot (BD Biosciences, San Diego, CA). Protein homogenates were prepared from *ksr*$^{+/+}$ and *ksr*$^{-/-}$ tissues, or MEFs in RIPA buffer and fractionated by SDS-PAGE (100 µg protein/lane). KSR expression was detected by western blot with a mouse monoclonal anti-KSR antibody (BD Biosciences, San Diego, CA). MEK and MAPK activation in MEFs were detected by western blot with anti-phospho-MEK and anti-phospho-MAPK specific antibodies (Cell Signaling, Beverly, CA).

Histology. Skin tissues were collected from 10-day old *ksr*$^{+/+}$, *ksr*$^{-/-}$ and *egfr*$^{-/-}$ (kindly provided by Dr. Laura Hansen) mice and fixed for 15-18 hours in 10% neutral buffered formalin, washed 2 hours in 70% ethanol and embedded in paraffin blocks. The blocks were sectioned 4-6 µm thick, placed on glass slides and stained with hematoxylin and eosin.

MEF studies. MEFs, derived from *ksr*$^{+/+}$ and *ksr*$^{-/-}$ day 12-13 embryos, were prepared as described[15]. 0.25 x 10^6 early passage MEFs (PDL<6) were seeded in 6-well plates and grown in DMEM supplemented with 10% FBS for 24 h at 37°C. After 48 h in serum-free medium, cells were stimulated with 0.05-1.0 ng/ml EGF for 3 min, washed with PBS and lysed in 0.2 ml of NP-40 lysis buffer (20 mM Tris-HCl, 137 mM NaCl, 2 mM EDTA, 10% Glycerol, 1% Nonidet P-40 plus protease and phosphatase inhibitors). To assess transformation capacity, MEFs from *ksr*$^{+/+}$ and *ksr*$^{-/-}$ mice were transduced sequentially with retroviral plasmids pWZL-Hygro-c-myc and pBabe-Puro-H-RasV12 (kindly provided by Scott Lowe, Cold Spring Harbor Laboratories), resuspended in 0.3% noble agar and seeded in 60 mm plates as described[15]. Colonies consisting of at least 50 cells were counted after 3 weeks.

Generation of Tg.AC/*ksr*$^{-/-}$ mice. Homozygous male and female Tg.AC transgenic mice[16] were obtained at 3-4 week of age from Charles River Laboratories Inc. (Wilmington, MA). To produce the target population, *ksr*$^{-/-}$ mice were first bred to hemizygous Tg.AC mice containing the *v*-Ha-*ras* transgene. The resulting F1 females

and males, heterozygous for *ksr* and hemizygous for the Tg.AC transgene, were then bred to obtain offspring in the *ksr* background. Nonresponder Tg.AC mice[17] were excluded from the study group. Presence of the Tg.AC transgene was determined by PCR amplification as follows: initial denaturation of 1 min 10 sec at 74°C, followed by 30 cycles with annealing at 55°C for 1min, extension at 72°C for 3min, and denaturation at 94°C for 1 min. The sequence of the Forward Primer was 5'-GGAACCTTACTTCTGTGGTGTGAC-3', and the sequence of the Reverse Primer was 5'-TAGCAGACACTCTATGCCTGTGTG-3'. PCR results were confirmed by Southern blot analysis as described[17].

Skin tumor experiments. Mice were treated twice weekly with 5 μg TPA (Sigma Chemical Company, St. Louis, Missouri) for 15 weeks and observed for papilloma development as described[16]. Offspring from the original Tg.AC mice in the FVB/N background from Charles River Laboratory were used as controls. Papillomas were counted weekly for 20 weeks. *v*-Ha-*ras* transgene expression in skin after TPA treatment was assessed by nested PCR as described[24].

Treatment with KSR-AS ODN. Murine KSR-AS ODN (5'-GCCTGGGATCTCCGTTTC-3'), KSR-sense ODN (5'-GAAACGGAGATCCCAGGC-3'), and a mismatch KSR-AS ODN (5'-GCAT GTGATC CCG TTGC-3') containing three nucleotide substitutions (in bold), were generated as phosphorothioate derivatives against nucleotides 214 to 231 of the unique CA1 domain (AAs 42-82) by Genelink Inc. (Hawthorne, NY). ODNs were gel purified, stored in TE buffer (10 mM TRIS, 1mM EDTA, pH 7.5), and diluted fresh in distilled sterile water for each experiment. Control ODN (5'-CACGTCACGCGCGCACTATT-3') was prepared similarly.

For *in vitro* studies, ODNs were delivered to A431 cells by Oligofectamine (Invitrogen). Briefly, $3x10^5$ cells, grown overnight in 6-well plates to 40-50% confluency, were switched to Opti-MEM (Invitrogen) medium 1 h prior to transfection, and incubated with a mixture of ODNs and Oligofectamine according to manufacturer's instructions. After 6 h, DMEM medium containing 30% FBS was used to adjust the FBS content to 10%. Cell proliferation was assayed at 96 h post-ODN treatment. For the matrigel invasion assay, cells were trypsinized 48 h after ODN treatment and assayed as described[25]

To determine the antitumor activity of KSR-AS ODN *in vivo*, 6 to 8-week-old male NCRNu mice (Taconic Inc., Germantown, NY) were transplanted subcutaneously into the right lateral flank with 50 mg of freshly-harvested and finely-minced A431 tumor that had been passaged through this strain. Mice were continuously infused with ODNs via Alzet osmotic minipumps for 5 days prior to tumor transplantation (to achieve steady-state plasma levels of ODNs by the Oligreen Assay) and for 36 days after transplantation. A 5.0 mg/kg body weight/day dose of ODN was chosen based on the range of 0.05–10 mg/kg body weight/day ODN commonly used for AS studies *in vivo*. Growing tumors were measured with calipers, and tumor volumes calculated using the following formula: volume (mm^3) = length x $width^2/2$.

Acknowledgements

We thank M.S. Soengas and S. Menéndez for assistance in the preparation and manipulation of MEFs. This work was supported by grant CA42385 from the National Cancer Institute (to R.K).

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Figure Legends

Fig. 1: Targeted disruption of the *ksr* gene in mice. *a*, Strategy for targeting the *ksr* allele. Simplified restriction maps of the 5' region of the wild-type *ksr* allele, the targeting vector, and the mutated allele are shown. Homologous recombination with endogenous *ksr* replaces an internal 1.1-kb *Sma*I-*Spe*I genomic fragment with a Neo cassette. *b*, Southern blot analysis of an ES clone showing the correct insertion of the targeting construct. Genomic DNA isolated from ES cells was digested with *Bgl*II and *Xho*I and hybridized to the 5' probe located just outside the 5'arm of the *ksr* targeting region as shown in *a*. The wild-type allele yields a 5.7-kb fragment whereas the mutant allele yields a 3.1-kb fragment. *c*, Genotyping of $ksr^{-/-}$ mice by PCR. The size of the PCR product is 493 bp for the wt allele and 312 bp for the mutated allele. *d*, Expression of *ksr* in wild type mouse embryos. The sizes of the two transcripts are 6.4 kb and 7.4 kb. *e*, Northern blot analysis of tissue *ksr* mRNAs. Poly-A^+ RNA, isolated from different tissues of adult $ksr^{+/+}$, $ksr^{+/-}$, and $ksr^{-/-}$ mice, was hybridized with a probe corresponding to domains CA2-CA4 in *ksr* cDNA. mRNA from NIH3T3 cells was used as control. *f*, KSR protein expression. Lysates prepared from wild-type and $ksr^{-/-}$ tissues were analyzed by western blot with a specific anti-KSR monoclonal antibody. Note that brain expresses the slightly shorter B-KSR1 isoform while lung and spleen express the longer KSR1 isoform. Lysates were also prepared from two independent sets of $ksr^{+/+}$ and $ksr^{-/-}$ MEFs. Equal loading was confirmed by reprobing blots with an anti-α-tubulin antibody.

Fig. 2: Skin phenotype in newborn $ksr^{-/-}$ mice. Full thickness skin cuts of 10-day old $ksr^{+/+}$, $ksr^{-/-}$ and $egfr^{-/-}$ mice were sectioned 4-6 μm thick, placed on glass slides, and stained with hematoxylin and eosin. s –serpentine, bl –blister, do-disoriented.

Fig. 3: Genetic and pharmacologic inactivation of KSR abrogates EGFR/Ras-mediated tumorigenesis *in vitro* and *in vivo*. *a*, Western blot analysis of MAPK activity upon EGF treatment. Low-passage MEFs derived from $ksr^{+/+}$ and $ksr^{-/-}$ were made quiescent by 48 h incubation in serum-free medium and stimulated with low doses of EGF for 3 min. Cells were lysed in NP40 buffer and activation of the MAPK cascade was examined by western blot with anti-phospho specific antibodies for the activated forms of MAPK(ERK1/2) and MEK1. Shown are representative blots from one of four

independent experiments. *b*, Proliferation of MEFs. 0.15×10^6 $ksr^{+/+}$ or $ksr^{-/-}$ low-passage MEFs were seeded on 60 mm plates and grown as described in Methods. Cells were trypsinized every other day and counted by hemacytometer. Data (mean±SD) are compiled from three independent experiments. *c*, RT-PCR detection of *v*-Ha-*ras* expression from total RNA isolated from the epidermis of Tg.AC/$ksr^{+/+}$ and Tg.AC/$ksr^{-/-}$ mice following TPA treatment. Intron spanning primers specific for the 3'UTR region of the *v*-Ha-*ras* transgene were used. The larger 279 bp amplicon, detected in the absence of reverse transcriptase [RT(-)], is derived from DNA and unspliced RNA. The smaller 214 bp amplicon is derived from spliced mRNA and is indicative of transgene expression. *d*, Mice, grouped according to genotype (10/group), were treated with 5 μg of TPA twice a week for 15 weeks. Papillomas were counted weekly for 20 weeks. *e*, KSR-AS ODN treatment inhibited A431 cell proliferation (left panel) and invasion (right panel). AS ODN treatment, cell proliferation assay and matrigel invasion assay were performed as described in Methods. For invasion, cells invading the matrigel were fixed, stained and counted from 10 randomly chosen fields. These results represent one of four independent experiments each. *f*, KSR-AS ODN treatment attenuated A431 tumor growth in nude mice. KSR-AS and Control ODNs were delivered via Alzet minipump over 6 weeks and tumor volumes determined as described in Methods. Data (mean±SD) are compiled from one of three independent experiments using 5 animals per treatment group.

<u>PANC-1 – Figure 4 – Figure 5</u>

Pancreatic ductal adenocarcinoma, the most common cancer of the pancreas, is notorious for its rapid onset and resistance to treatment. The 5-year survival is a dismal 3%. K-*ras* is the most commonly mutated oncogenes in human cancers. Activating mutations within the K-*ras* gene have been identified in up to 90% of pancreatic carcinomas. The high frequency of K-*ras* mutations in human pancreatic tumors suggests that constitutive Ras activation plays a critical role during pancreatic oncogenesis. In order to evaluate the effectiveness of KSR-specific antisense oligonucleotide (AS-ODN) in inhibiting human pancreatic tumorigenesis, we employed the well-established human pancreatic tumor cell line PANC-1 which harbors a codon 12 K-*ras* mutation (GGT? GTT) for our study. *In vitro*, treatment of PANC-1 cells with KSR AS-ODN resulted in a dose-dependent inhibition of cell proliferation and invasion through Matrigel (80% and 70% inhibition

respectively at 5 μM). *In vivo* studies are undergoing to evaluate the efficacy of AS-ODN in abrogating PANC-1 tumorigenesis in athymic mice. We are also expanding our *in vitro* screening to a panel of human pancreatic cell lines harboring the same codon 12 mutation or different oncogenic K-*ras* mutations.

Figure 4: PANC-1 proliferation assay

PANC-1 cells were seeded at 2 X 10^5 cells/well in the 12-well plate and transfected with AS and control ODNs as described in the Nature Medicine manuscript. 96 h after treatment, cells were trypsinized counted. SC: nonsense sequence control ODN. S: control ODN with sense sequence to the AS ODN. AS: antisense ODN.

Figure 5: PANC-1 invasion assay: PANC-1 cells were seeded at 2 X 10^5 cells/well in the 12-well plate and transfected with AS and control ODNs as described in the Nature Medicine manuscript. 96 h after treatment, cells were trypsinized and 2000 cells were added to each transwell coated with 45 μg of Matrigel and the invasive capacity of PANC-1 cells was assessed by the Matrigel invasion assay as described. Cells invaded were fixed, stained and counted under 40 X. NT: non-treated. SC: nonsense sequence control ODN. S: control ODN with sense sequence to the AS ODN. AS: antisense ODN.

Fig 1a-

a

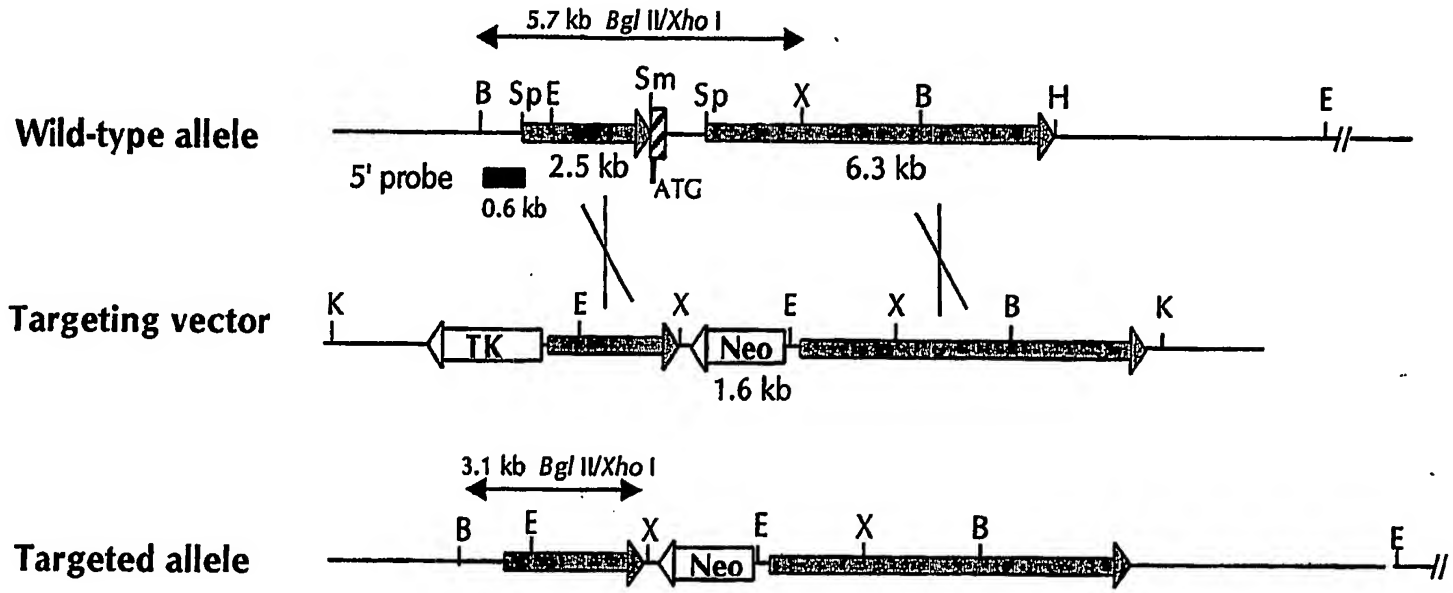


Fig 1b
b

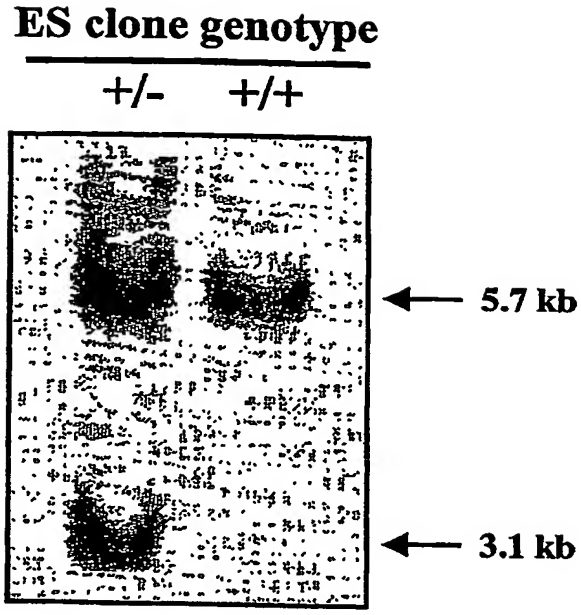


Fig 1c
c

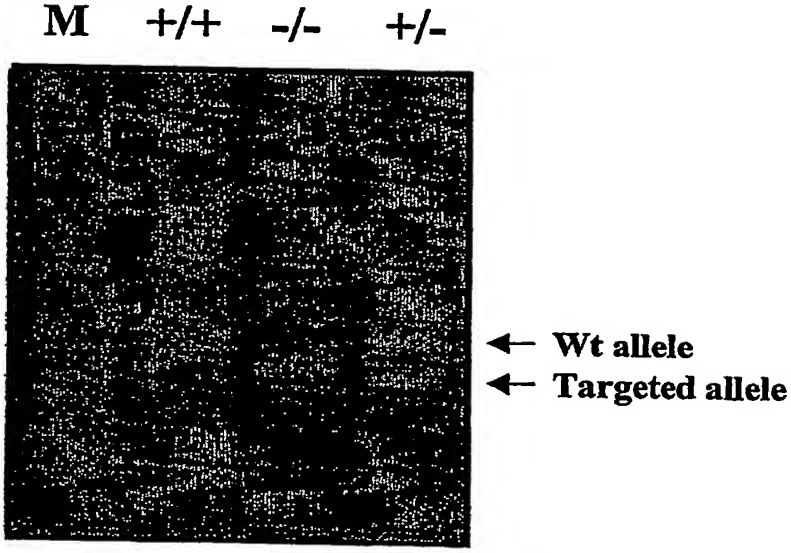


Fig 1d

d

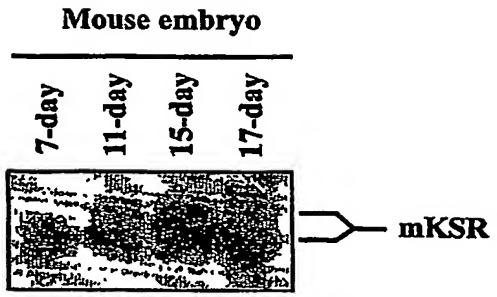


Fig 1e

e

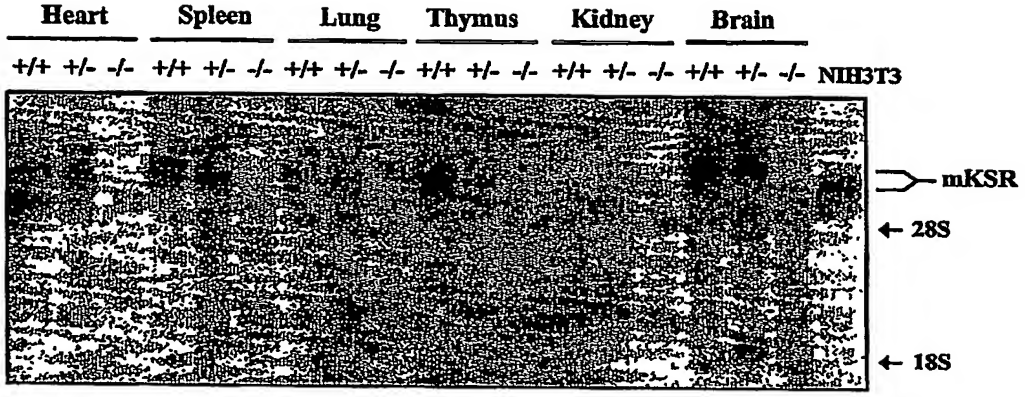


Fig 1f

f

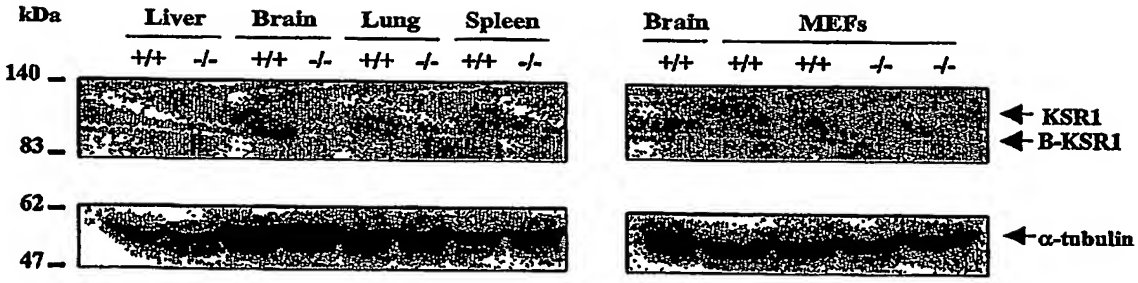


Fig 2

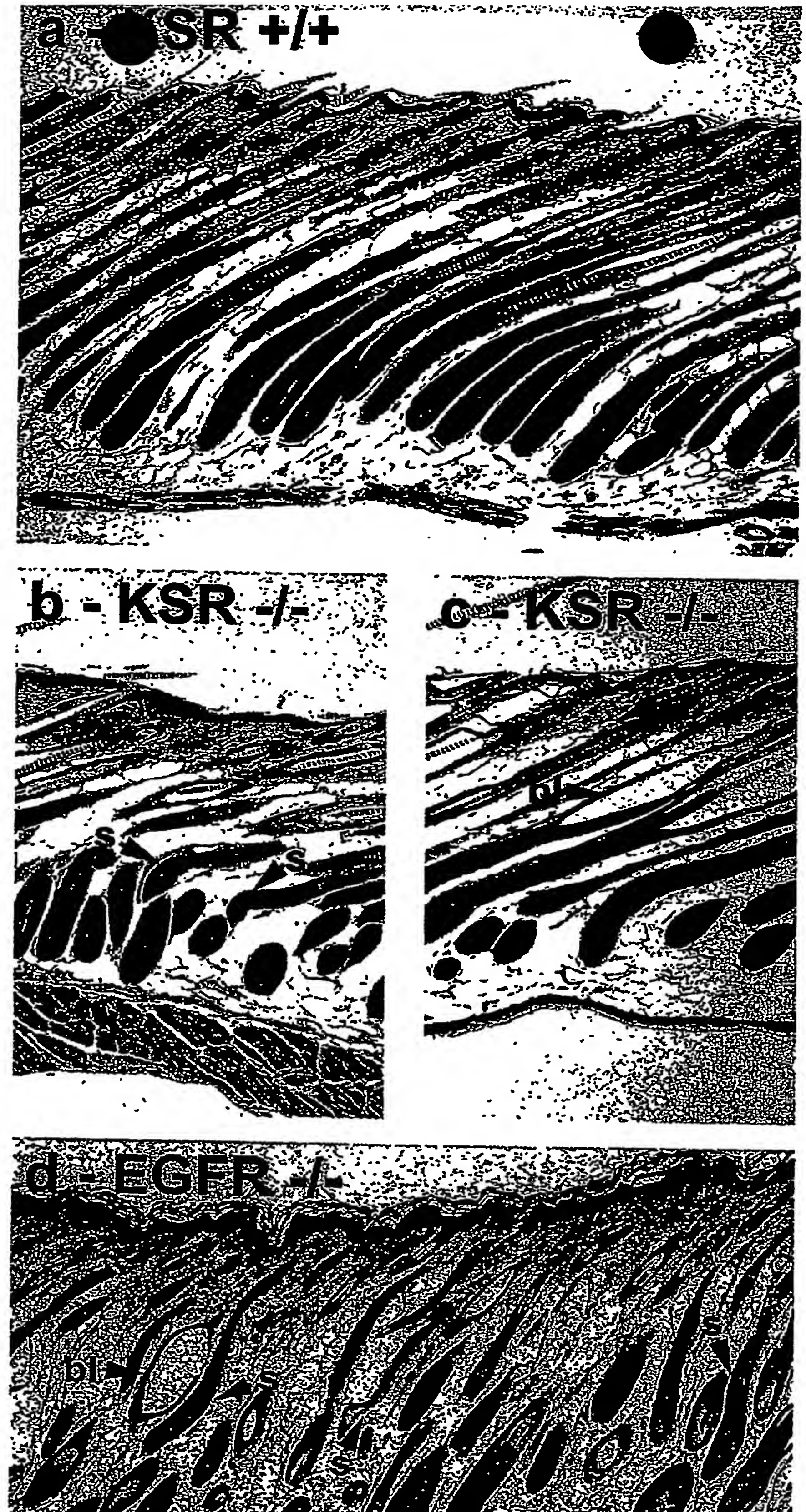


fig 3a

a

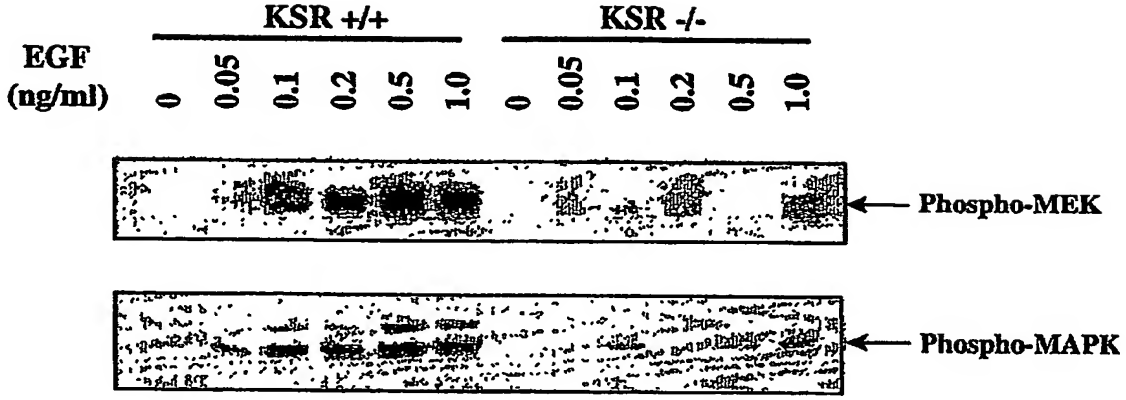


fig 3b

b

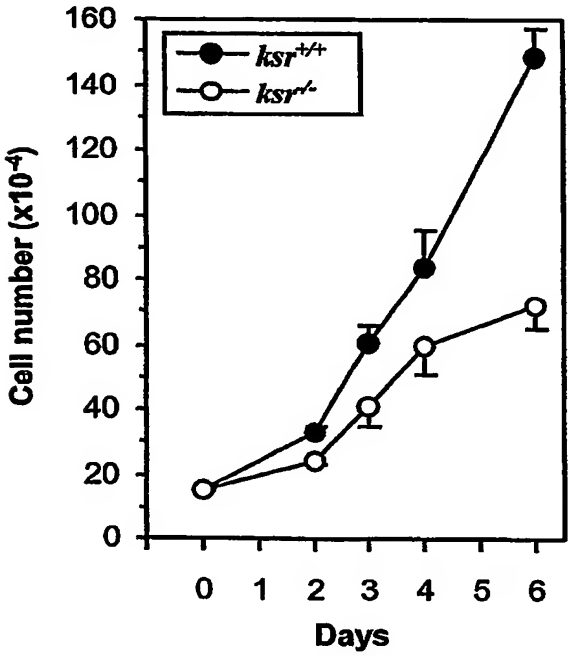
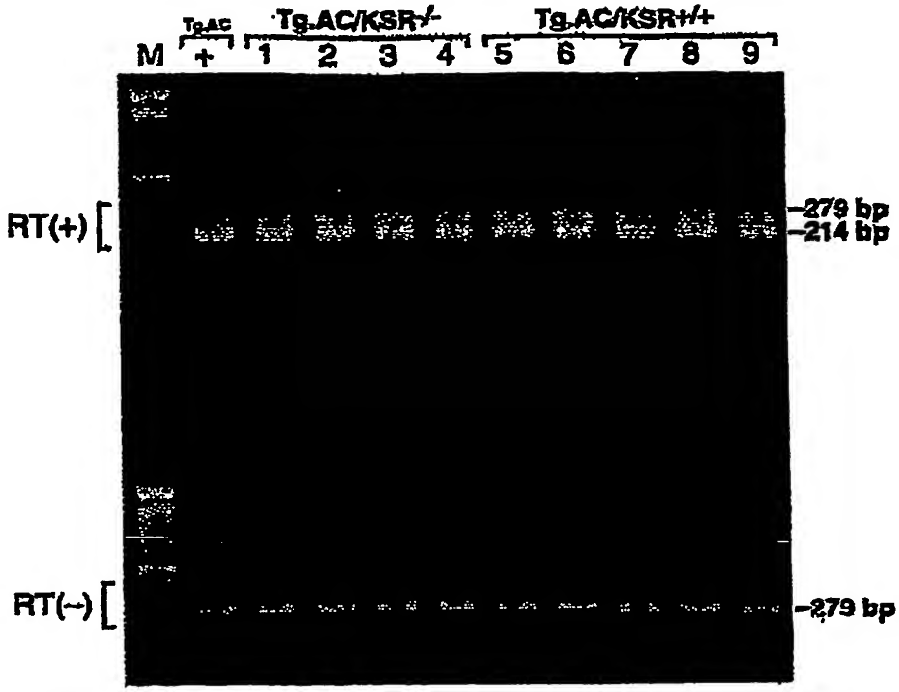


fig 3c.

C



c

fig 3 d -

d

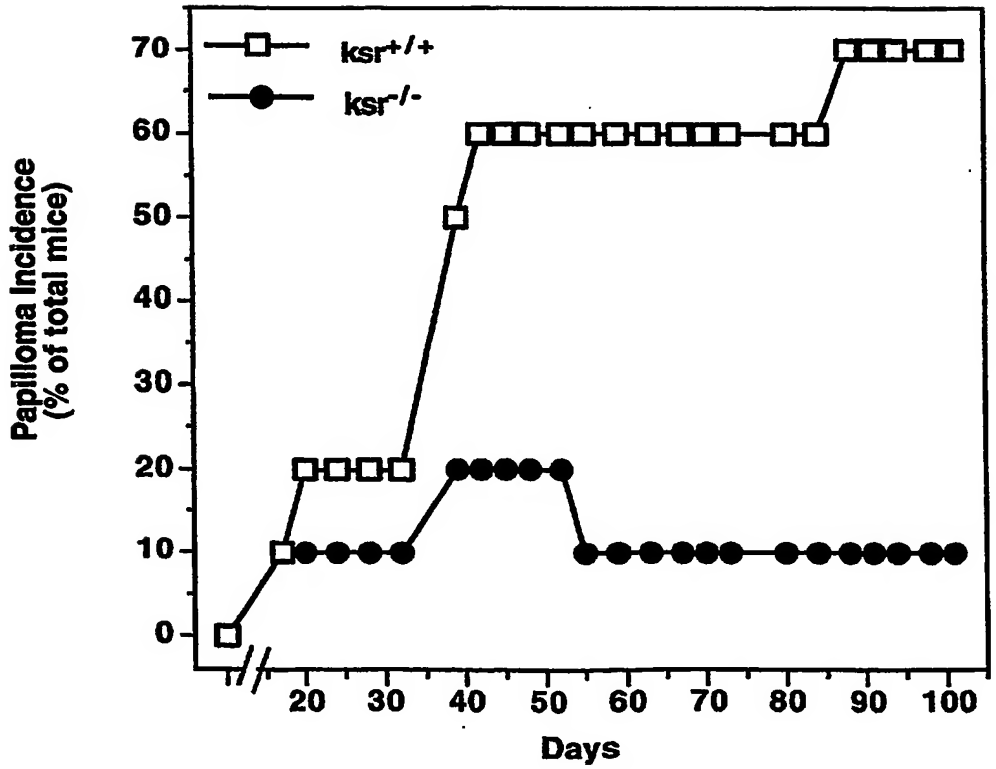

ksr+/+
ksr-/-
Papilloma Incidence
(% of total mice)
0
10
20
30
40
50
60
70
20
30
40
50
60
70
80
90
100
Days

fig 3e

e

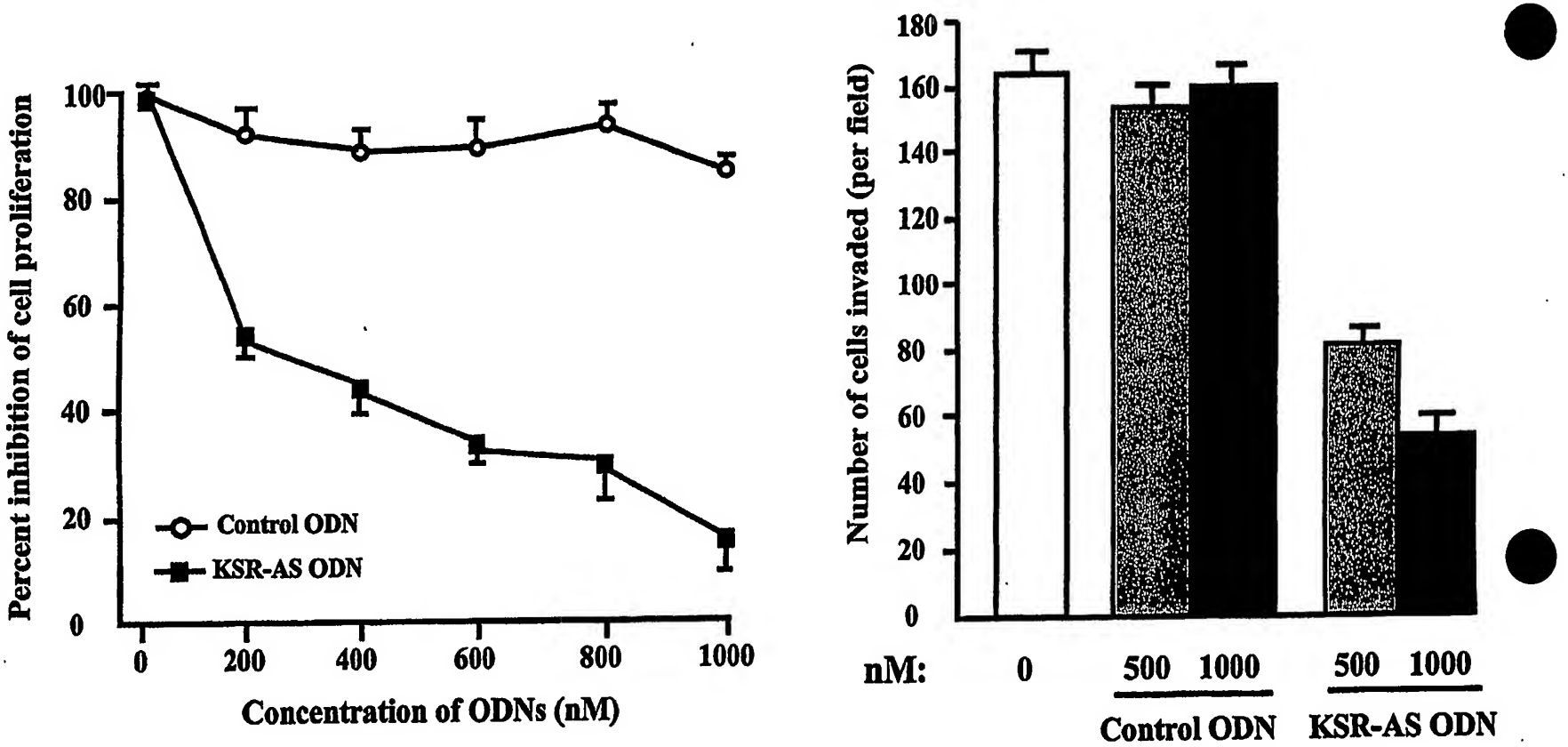


Fig 3f

f

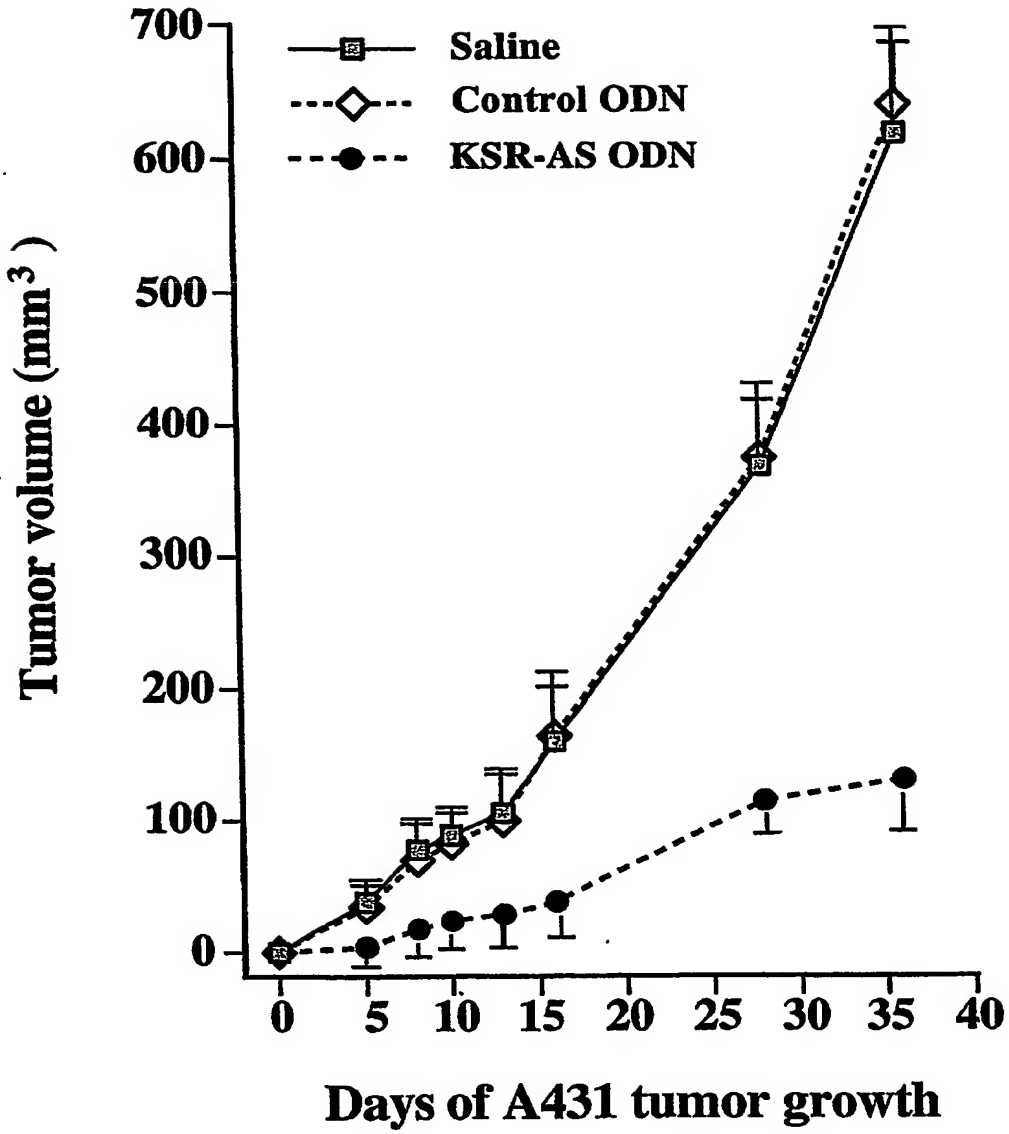

Saline
Control ODN
KSR-AS ODN
Tumor volume (mm3)
700
600
500
400
300
200
100
0
0
5
10
15
20
25
30
35
40
Days of A431 tumor growth

Fig 4

KSR AS ODN inhibited human pancreatic cancer cell PANC-1 proliferation

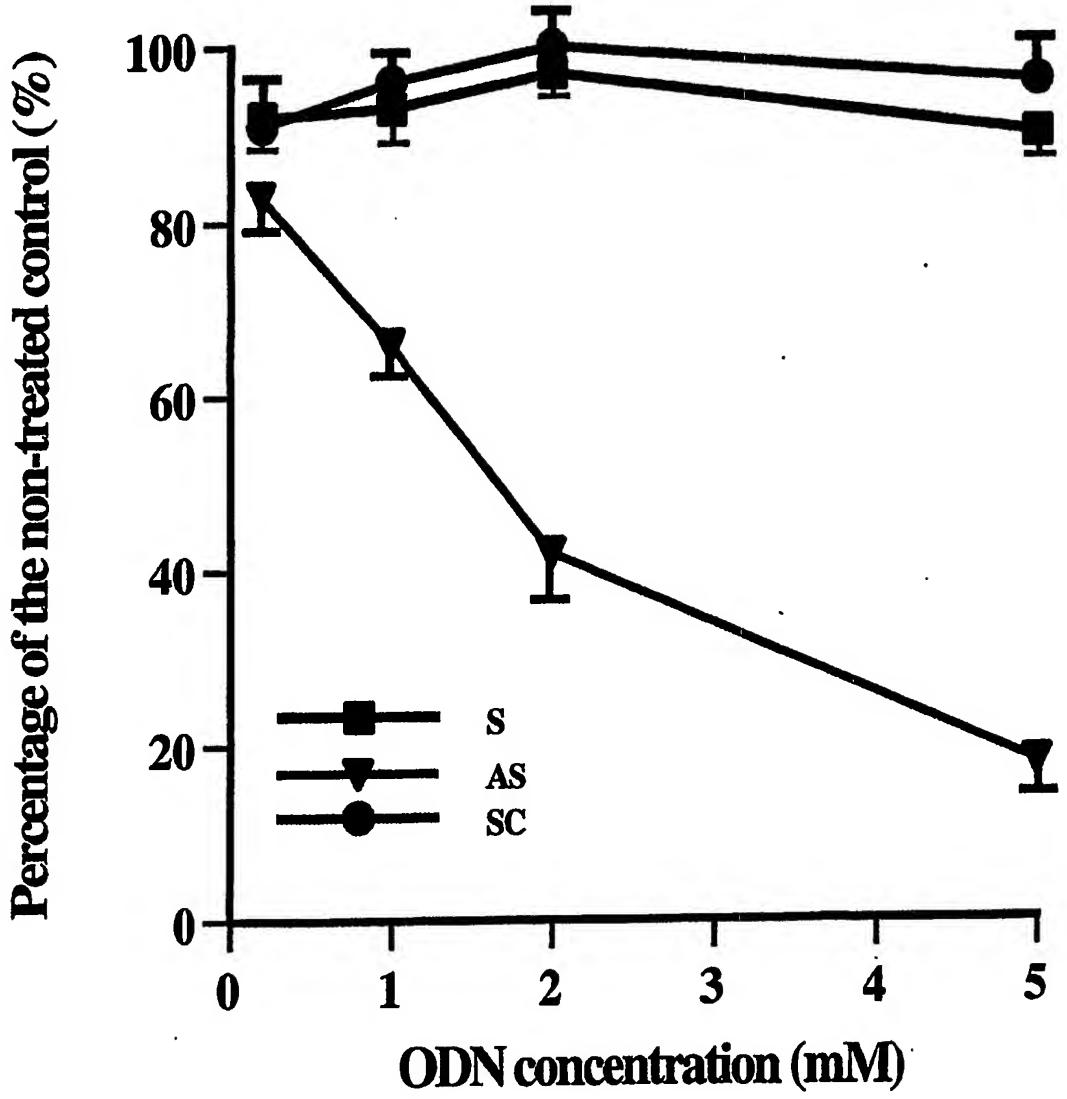
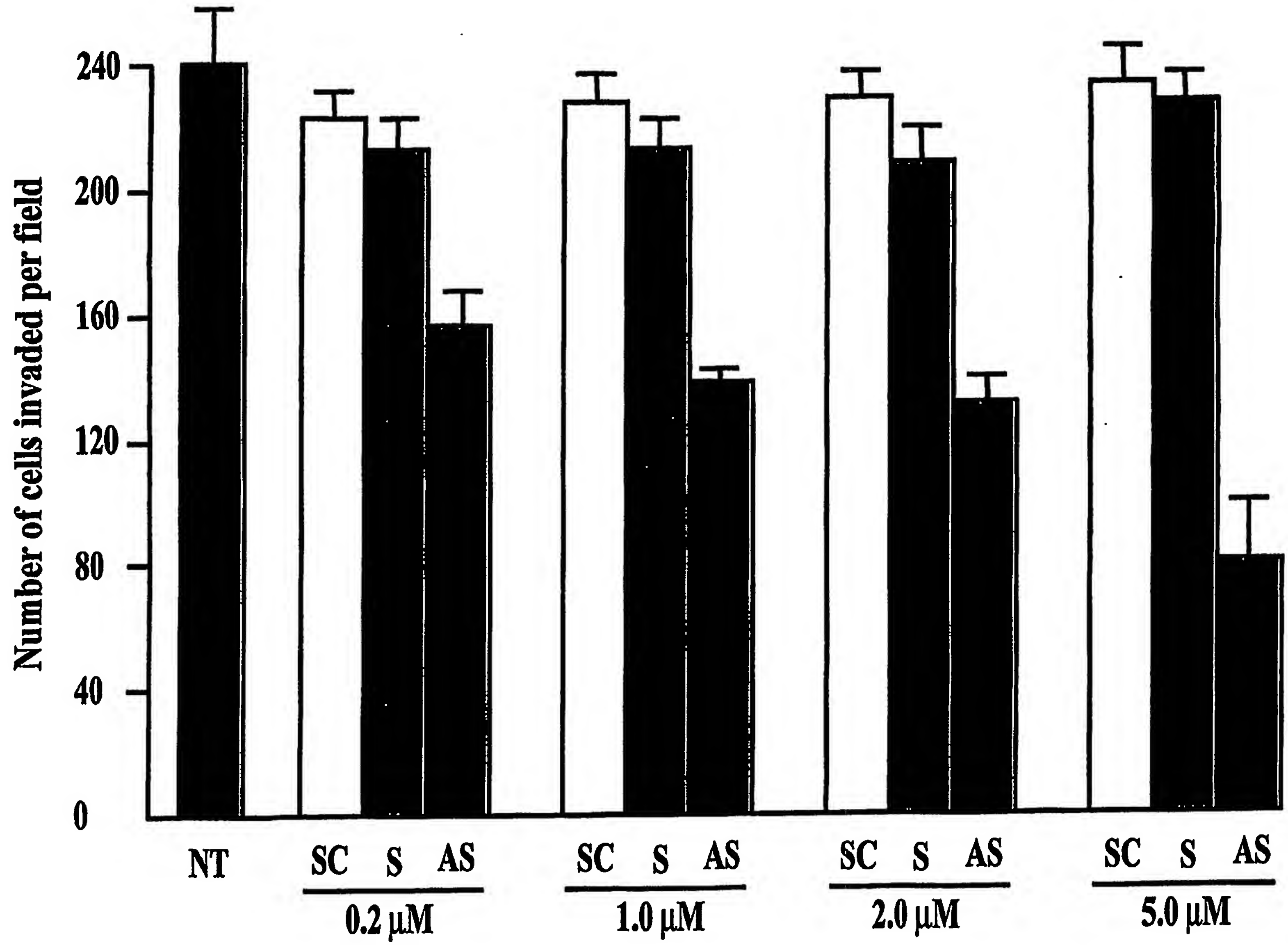


Fig 5

KSR AS ODN inhibited human pancreatic cancer cell PANC-1 invasion



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